VIROLOGY 211, 507–515 (1995)

Effect of Non-*H-2*-linked Genes on Anti-virus Immune Responses and Long-Term Survival in Mice Persistently Infected with E-55+ Murine Leukemia Virus

NILI AVIDAN,* KATHLEEN M. TUMAS-BRUNDAGE,† THOMAS G. SIECK,*
MICHAEL B. PRYSTOWSKY,‡ and KENNETH J. BLANK*,[1]

*Department of Pathology and Laboratory Medicine, Medical College of Pennsylvania and Hahnemann University, Philadelphia, Pennsylvania 19102; †Jefferson Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107; and ‡Department of Pathology, Albert Einstein College of Medicine/ Montifiore Medical Center, Bronx, New York 10462

Received February 22, 1995; accepted June 16, 1995

We have previously demonstrated that BALB/c-H-2^k (BALB.K) mice are susceptible to the development of thymic lymphoma induced by E-55+ murine leukemia virus (MuLV). In the present studies, C57BL/10-H-2^k (B10.BR) mice were found to be resistant to E-55+ MuLV-induced lymphoma despite the fact that these mice become persistently infected. This resistance to lymphomagensis is mediated by the anti-virus immune response since immunosuppressed mice progress to develop disease. The protective immune response in B10.BR mice is bimodal with respect to time after virus infection. The early immune response results in a dramatic decrease in the number of virus-infected cells within 4–8 weeks after infection. This decrease in virus-infected cells occurs in immunocompetent mice from strains that are either resistant (B10.BR) or susceptible (BALB.K) to E-55+ MuLV-induced disease. Subsequently, susceptible mice develop an increase in infected cells, whereas no increase in infected cells occurs in resistant mice despite the fact that they are persistently infected. This later phase of resistance in B10.BR appears to be mediated by T cells. Since B10.BR and BALB.K both express the *H-2*^{k} haplotype, resistance appears to be mediated by a non-*H-2*-linked gene(s). (BALB.K × B10.BR)F_1 mice are resistant to disease development, indicating resistance is a dominant trait.

INTRODUCTION

Common patterns in the course of HIV infection have been observed among infected individuals (Pantaleo *et al.*, 1993a). Initially, HIV infection is associated with high levels of virus production resulting in viremia, followed by a period 1–12 weeks after infection when an anti-virus immune response is generated (Daar *et al.*, 1991; Clark *et al.*, 1991). This immune response is associated with a dramatic decline in viremia although virus can still be detected in the plasma by sensitive assay techniques (Piatak *et al.*, 1993; Pan *et al.*, 1993). Virus persists, however, despite the presence of this immune response, most notably in the lymph nodes (Pantaleo *et al.*, 1993b) where infected cells appear to be sequestered. This persistent infection eventually results in progression to AIDS.

In the years since HIV has been identified as the etiological agent in AIDS, it has become apparent that different cohorts of HIV-infected individuals exist. One cohort includes individuals who are considered long-term survivors, that is, those that have survived an HIV infection for greater than 10 years without overt symptoms of virus-induced disease (Ezzell, 1993). Investigations of these individuals have found associations between host genetic factors and long-term survival (Steel *et al.*, 1988; Kaslow and Mann, 1994; Ezzell, 1993). However, the genetic basis for long-term survival in persistent retrovirus infection remains poorly understood.

To facilitate the analysis of the influence of genetic background on the relationship between long-term survival and the establishment of a persistent retrovirus infection, we have utilized a murine leukemia virus, E-55+ MuLV, as a murine model for persistent HIV infection. E-55+ MuLV infection in mice shares several characteristics with HIV infection in humans (Tumas *et al.*, 1993a). Primary E-55+ MuLV infection results in a large number of virus-infected cells in the spleen and bone marrow 1–2 weeks after infection. One to 4 weeks after infection, an anti-virus immune response is generated, resulting in a dramatic decrease in the number of virus-infected cells similar to the decrease in viremia which occurs shortly after HIV infection. However, despite this immune response in both E-55+ MuLV-infected mice and HIV-infected humans, a persistent virus infection is established with infected cells sequestered in lymphoid tissue (Pantaleo *et al.*, 1993b; Pan *et al.*, 1993; Tumas *et al.*, 1993a).

In the present studies, we have determined that certain inbred strains of mice are susceptible (progressors) or resistant (long-term survivors) to E-55+ MuLV-induced thymic lymphoma despite the fact that mice from both these phenotypes are persistently infected. Specifically, susceptible BALB/c-H-2^k (BALB.K) mice and resistant

[1] To whom correspondence and reprint requests should be addressed. Fax: (215) 246-5918. Internet : blankk@hal.hahnemann.edu.

0042-6822/95 $12.00

C57BL/10-H-2^k (B10.BR) mice develop a persistent infection upon inoculation with E-55+ MuLV but only BALB.K mice progress to develop lymphoma. Although both BALB.K and B10.BR mice are able to generate an early anti-virus immune response which results in the decrease in the number of virus-infected cells, the basis for long-term survival in E-55+ MuLV-infected B10.BR mice appears to be their ability to generate a late virus-specific T cell response that is not observed in progressor BALB.K mice. These differences between these strains appear to be regulated by non-*H-2*-linked genes since both strains express the *H-2*^{k} haplotype.

The genetic regulation of the immune response to murine leukemia viruses has been studied extensively using Friend murine leukemia virus (FV). Unlike E-55+ MuLV, which causes disease with a long latency period, FV is an acute transforming virus which results in detectable neoplastic growth of erythroid precursor cells within 9 days of infection. Previous studies have demonstrated that recovery from FV-induced disease is mediated by T cells and antibody. Anti-FV T cell responses are regulated by two genes linked to the *H-2* complex, *Rfv-1,* and *Rfv-2* (Chesebro and Wehrly, 1978; Britt and Chesebro, 1980; Miyazawa *et al.,* 1992a, b), whereas protective antibody responses are regulated by a non-*H-2*-linked gene, *Rfv-3* (Doig and Chesebro, 1979; Chesebro *et al.,* 1979; Chesebro and Wehrly, 1979).

In contrast to the observations regarding genetic control of the anti-virus immune response in FV-infected mice, our present studies demonstrate that differences between the T cell response to E-55+ MuLV in resistant and susceptible mice is regulated by a non-*H-2*-linked gene(s). In addition, antibody responses to E-55+ MuLV in susceptible BALB.K and resistant B10.BR mice are similar, suggesting that *Rfv-3* plays no role in protection from E-55+ MuLV-induced disease despite the fact that the *env* sequence of E-55+ MuLV is highly homologous to the *env* sequence of F-MuLV, the helper virus component of FV (Tumas *et al.,* 1993b).

MATERIALS AND METHODS

Mice. Male and female B10.BR mice 6–8 weeks old were purchased from Jackson Laboratories (Bar Harbor, ME). BALB.K were obtained from our breeding colony.

Virus. E-55+ MuLV virus was isolated from a BALB.K leukemic spleen (Pozsgay *et al.,* 1989) and was biologically cloned by limiting dilution on Sc-1 cells. The virus used in these studies was propagated on Sc-1 cells.

Virus-specific PCR and Southern blot assay. Adult mice were inoculated ip with 10^4 focus-forming units (FFU) E-55+ MuLV per mouse and sacrificed at various time points. Genomic DNA was isolated from the bone marrow of infected mice as previously described (Tumas *et al.,* 1993a). One microgram of genomic DNA was amplified by PCR in 10× PCR buffer, 0.2 *mM* of dATP, dCTP, dGTP, dTTP, 0.2*M* of each primer, and 3 U of TAQ DNA polymerase (Boehringer Mannheim, Indianapolis, IN) in a final volume of 100 μl. The primers used (5′ primer: GGATCCACGCTGCCCACG; 3′ primer: GATTAAGAATGCAGGGTC) specifically amplified the entire E-55+ MuLV *env* region and produced a 2-kb fragment. As a positive control, an endogenous virus fragment (0.85 kb) was amplified by PCR in a separate PCR reaction using a different set of primers (5′ primer: GGATCCACGCTGCCCACG; 3′ primers: GATCCGAGGTCCTAGATTT). PCR amplifications were carried out under the following amplification conditions: 92° for 2 min, 55° for 1 min, 72° for 2 min for 30 cycles, and then 72° for 10 min and soak at 25° until removed from the instrument. Genomic DNA from 1 week infected and uninfected B10.BR and BALB.K mice were used as positive and negative controls, respectively.

Twenty microliters of each PCR reaction was separated by electrophoresis on a 0.8% TBE agarose gel. Gels were depurinated in 0.25 *M* HCl for 30 min, denatured in 0.5 *M* NaOH and 1 *M* NaCl for 30 min, and neutralized with 0.5 *M* Tris–HCL, 3 *M* NaCl, pH 7.5 for 30 minutes. The gels were blotted onto Nytran membrane overnight using 20× SSC as transfer buffer. The E-55+ MuLV *env* gene probe was labeled with P^{32}dCTP using a random primer labeling Kit (Boehringer Mannheim, Indianapolis, IN). This probe consisted of the entire *env* sequence cloned from E-55+ MuLV. The unincorporated radioactivity was separated using G-50 Quick-spin columns (Boehringer Mannheim). As a control, 1 μg of Lambda DNA digested with *Eco*RI and *Hin*dIII was also labeled with [P^{32}]dCTP and used as a probe to hybridize to molecular weight (MW) markers allowing us to ensure that the DNA had transferred from the gel to the blots. The blots were hybridized overnight, washed as previously described (Tumas *et al.,* 1993a), and were exposed to X-ray film for 24 hr.

Flow cytometry. Single-cell suspensions were prepared from spleens of mice inoculated with E-55+ MuLV. The cells were washed twice with HBSS buffer and overlaid onto Sc-1 fibroblast cells which were plated 1 hr previously at a concentration of 5×10^3 cells/well in a 24-tissue culture plate. After inoculation for 1 hr at 37°, the wells were washed twice with warm DMEM and the fibroblasts incubated at 37° in a 5% CO_2 incubator for 10 to 14 days. For flow cytometry, the fibroblasts were removed from the plates by trypsinization. Cells were washed and resuspended at a concentration of 10^6 cell/ml and incubated with monoclonal antibodies (mAb) against gp70 virus envelope, m55, m307, m350 for 1 hr on ice (Pozsgay *et al.,* 1989). Hybridoma cells producing these mAb were a gift of Dr. Bruce Chesebro (Chesebro *et al.,* 1983, 1981). The cells were washed three times and incubated on ice for 1 hr with FITC conjugate anti-mouse-Ig antibody. After incubation with the second FITC-labeled antibody, cells were fixed with 0.1% para-

formaldehyde. Fluorescence was analyzed by flow cytometery.

Adoptive transfer and T and B cell depletion. Depletion of donor splenic lymphocyte population was performed by treatment of spleen cells with antibody and complement (C′) followed by three cycles of panning. After the panning procedure nonadherent cells were inoculated into lethally irradiated (900R) recipient mice. Initial depletion of T and B lymphocytes utilized anti-CD3 mAb (produced by YCD3 hybridoma cells) (Portoles *et al.*, 1989) and anti-Ia^k alloantiserum. Donor cells were incubated with antibody for 1 hr at 4° followed by addition of 10% baby rabbit C′ and incubation for 1 hr at 37°. Cells were then washed three times in PBS. For further purification by panning, plates were coated with 20 μg of antibody per cm^2 at 4° overnight and blocked with 1% BSA for 2 hr at 4°. Depletion of T and B cells was performed by three cycles of panning using anti-Thy1.2 and anti-Ig antibodies, respectively. For each cycle of panning, cells were incubated in the antibody-coated plates for 30 min at 4°. The nonadherent cells were harvested, resuspended, and transferred to new antibody-coated plates to perform another cycle. After three cycles, the cells were washed twice in PBS and resuspended at a concentration of 5×10^7 cells/ml. Depleted spleen cells contained <5% T or B cells as determined by flow cytometry. Donor bone marrow cells were harvested from mice inoculated with 10^4 FFU of E-55+ MuLV 8 weeks previously and depleted of T and B cells as described above for spleen cell depletions. Recipient mice were inoculated iv with 5×10^6 bone marrow cells from infected mice (as a source of virus-infected cells) mixed with 5×10^6 spleen cells (as a source of effector cells). Recipient mice were sacrificed 4 weeks after cell transfer followed by isolation of genomic DNA from their bone marrow and PCR analysis performed to detect presence of provirus as described above.

Antibody-mediated cytotoxicity. In the antibody-mediated cytotoxicity assay, KgV cells (an E-55+ MuLV-induced tumor cell line) were used as targets (Kłyczek and Blank, 1989). These target cells were radiolabeled by incubation with 200 μC $^{51}Cr(Na_2CrO_4)$ for 1 hr at 37°. After labeling, target cells were washed three times and resuspended at a concentration of 2×10^6 cells/ml. Fifty microliters of a serial twofold dilution of antiserum was mixed with 50 μl of labeled target cells and incubated for 30 min at 37°. A 1:4 dilution of baby rabbit C′ was then added to each well and incubated 45 min at 37°. The plates containing the assay were centrifuged, 100 μl of supernatant was removed, and released radioactivity was measured in a gamma counter. Control wells contained targets plus complement or medium without antibody. Percentage of lysis was calculated as [(cpm released after incubation with antibody and C′ - cpm released after incubation with medium alone) divided by (total releasable cpm)] $\times$ 100.

RESULTS

In previous studies, BALB.K mice were found to be highly susceptible to thymic lymphoma induced by E-55+ MuLV (Tumas *et al.*, 1993a). Mice from this strain developed a high incidence (100%) of T cell lymphoma within 4–7 months after inoculation with E-55+ MuLV. In the present study, mice from other strains were inoculated with E-55+ MuLV to determine if animals resistant to E-55+ MuLV could be identified. The results demonstrated that B10.BR mice were resistant to E-55+ MuLV-induced lymphoma since no mice from this strain developed lymphoma within 9 months of infection (Table 1). Ten of these E-55+ MuLV-infected B10.BR mice were observed for over 24 months without lymphoma development. (B10.BR $\times$ BALB.K)F1 mice were also resistant to virus-induced lymphomagenesis, indicating that the resistance expressed by the B10.BR parent was a dominant trait. In contrast to immunocompetent B10.BR mice, B10.BR mice immunosuppressed by sublethal irradiation (550R) demonstrated a high incidence (100%) of E-55+ MuLV-induced lymphoma 4–7 months after infection. Sc-1 cells incubated with cell-free extracts produced from the lymphomatous spleens of immunosuppressed B10.BR mice expressed virus epitopes that bound mAb 55, 307, and 350, which are encoded by E-55+ MuLV but not endogenous ecotropic, xenotropic, or RadLV retroviruses (data not shown) (Chesebro *et al.*, 1983, 1981). In addition, tumor cell lines cultured from the spleens of these lymphomatous B10.BR mice were found both to bind mAb 55, 307, and 350 and to contain provirus DNA that hybridized to an E-55+ MuLV-specific probe (data not shown). Thus, it appears that these lymphomas from immunosuppressed mice were induced by E-55+ MuLV and not an endogenous virus induced by the immunosuppressive radiation treatment.

These data demonstrated that the difference in the susceptibility to EE-55+ MuLV-induced lymphomagenesis between BALB.K and B10.BR mice is the result of differences in alleles of non-*H-2*-linked background genes expressed by these two strains of mice since both strains express the same *H*-2^k haplotype (Klein, 1973). In addition, the difference in susceptibility exhibited by immunocompetent and immunocompromised mice indicates that resistance to E-55+ MuLV-induced lymphomagenesis in B10.BR is related to the anti-virus immune response generated in B10.BR mice.

Our previous studies demonstrated that inoculation of adult BALB.K with E-55+ MuLV results in persistent retrovirus infection (Tumas *et al.*, 1993a). Initial infection of BALB.K mice resulted in an acute phase (up to 4–8 weeks after infection), during which cells containing provirus were readily detectable by PCR analysis. This acute phase was followed by a preleukemic phase in which cells containing provirus declined to undetectable background levels. However, the emergence of leukemic cells

TABLE 1

Incidence of E-55+ MuLV-Induced Lymphoma in B10.BR, BALB.K, and F_1 Mice

Strain	MHC	Untreated	Sublethal irradiation (550R)
B10.BR	H-2^k	0/30[a,b] (0%)[c]	20/20 (100%)
(B10.BR × BALB.K)F1	H-2^k	0/10 (0%)	ND
BALB.K	H-2^k	30/30 (100%)	20/20 (100%)

Note. Mice were immunosuppressed by sublethal irradiation (550R). Irradiated and untreated mice were inoculated ip with 10^4 focus forming units (FFU) of E-55+ MuLV per mouse. Mice were observed for the development of lymphoma by abdominal palpation at various intervals and splenic enlargement was recorded on sale of 0 (normal) to +3 (7- to 10-fold increase over normal). Mice were classified as lymphomatous when spleens were determined to be +2. Observations were made over a period of 9 months after inoculation of E-55+ MuLV. ND, not determined.

[a] Number of lymphomatous mice/total number of mice in the group.

[b] Ten B10.BR mice were observed for an extended period of 2 years.

[c] Percentage of mice positive for lymphoma.

4–7 months after infection that contain the E-55 genome indicated that these mice were persistently infected. Since B10.BR mice were resistant to E-55+ MuLV-induced lymphoma, studies were performed to determine if this resistance was associated with complete elimination of virus-infected cells. Initial analysis for the presence of E-55+ MuLV-infected cells in B10.BR mice was performed by PCR as previously described (Tumas *et al.*, 1993a). B10.BR and BALB.K mice were sacrificed at various time points after infection, bone marrow cells harvested, and genomic DNA analyzed by PCR for E-55+ MuLV provirus using E-55+ MuLV-specific primers that produced a 2-kb product. The results demonstrated that E-55+ MuLV provirus was readily detectable in hematopoietic tissues of immunocompetent B10.BR and BALB.K mice 2 weeks after virus inoculation (Fig. 1A). This acute phase was followed by a period in which cells containing provirus were not detectable above background levels (Figs. 1B and 1C). By 32 weeks after infection E-55+ MuLV provirus was detectable in BALB.K but not B10.BR bone marrow (Fig. 1D). We have not observed a phase in B10.BR mice correlating with the leukemic phase in BALB.K mice in which a subsequent increase in the number of virus-infected cells occurs over a period of 24 months.

Although PCR analysis failed to detect the presence of infected cells in B10.BR mice inoculated with E-55+ MuLV, it appeared possible that these mice were still persistently infected at low levels. To test this possibility, B10.BR bone marrow was harvested 8 weeks after infection, treated with anti-CD3 + C′ to remove T cells, and transferred into lethally irradiated (900R) syngeneic mice. Prior to transfer, B10.BR donor bone marrow (Fig. 2, lane 3) and spleen (Fig. 2, lane 4) did not contain detectable levels of proviral DNA by PCR analysis, indicating that if virus-infected cells were present, they were at a level below the sensitivity of the assay (<1 in 10^4 cells) (Tumas *et al.*, 1993a). The rationale for this procedure was that infected donor cells would produce virus in the immunocompromised host, which, in the absence of an anti-virus immune response, would result in the spread of the virus to uninfected cells. The subsequent amplification of virus in the bone marrow recipient would allow its detection. Bone marrow cells were harvested from recipient mice 4 weeks after transfer and tested for E-55+ MuLV provirus by PCR analysis. The results demonstrated that virus-infected cells were readily detectable in the recipient mice indicating that donor bone marrow from infected B10.BR mice contained virus-infected cells (Fig. 2, lanes 1 and 2). The virus detected in the recipient mice was determined to be E-55+ MuLV and not endogenous virus by the fact that infected cells bound mAbs 55, 307, and 350, which are not expressed by endogenous virus (data not shown) (Chesebro *et al.*, 1983, 1981). In addition, the PCR primers used to detect virus were specific for E-55+ MuLV and did not produce an E-55+ MuLV-specific 2-kb band when PCR was performed with genomic DNA from uninfected mice (Fig. 2, lane 5)(bands of other sizes are occasionally seen in uninfected animals, indicating the presence of endogenous sequences with some homology to E-55+ MuLV).

The pattern of infection observed in immunocompetent B10.BR mice differed from that observed in immunosuppressed mice. As described above, immunocompetent B10.BR mice have detectable provirus during the acute phase of infection which subsequently declines to background levels. In contrast, provirus remained detectable in immunosuppressed mice throughout the infection (Fig. 3). Eight weeks after virus inoculation, no provirus was detected in any of five immunocompetent B10.BR mice (Fig. 3, Lanes 1–5) by PCR analysis, whereas four of five immunosuppressed mice were positive (Fig. 3, Lanes 6–10). The fact that immunosuppressed mice showed no decrease in virus-infected cells after the acute phase of infection strongly suggests that an anti-virus immune response is responsible for the decrease in virus-infected cells observed in immunocompetent mice.

To examine the role of the immune response in maintaining persistent infection at a low level in B10.BR mice, adoptive transfer experiments were performed to deter-

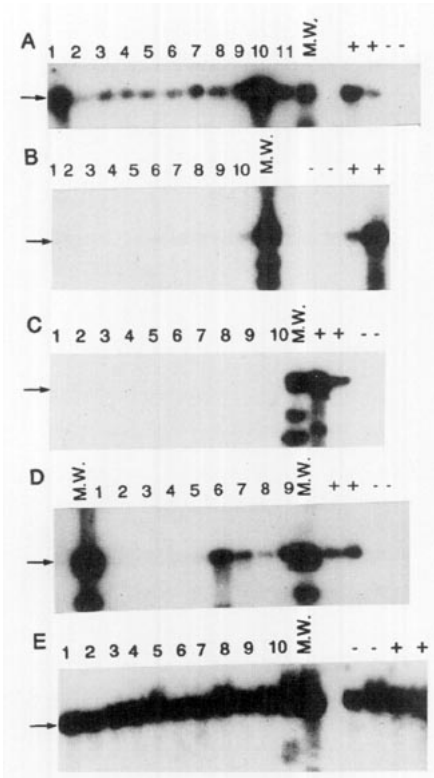

FIG. 1. Detection of E-55+ MuLV provirus in bone marrow cells from B10.BR and BALB.K mice at various intervals after infection. Bone marrow cells from mice inoculated with 10^4 FFU of E-55+ MuLV were harvested at various intervals after infection. The presence of cells with provirus was determined by PCR analysis using E-55+ MuLV-specific primers as described under Material and Methods. E-55+ MuLV *env* (2 kb) was amplified by PCR using virus-specific primers from 1 μg of genomic DNA. Twenty microliters of the PCR reaction was separated on an 0.8% agarose gel, blotted onto Nytran membrane, and hybridized overnight with E-55+ MuLV-specific probe. (A) Bone marrow cells from mice 2 weeks after infection. Lanes 1–6, B10.BR mice; Lanes 7–11, BALB.K mice. (B) 8 weeks after infection. Lanes 1–5, B10.BR mice; Lanes 6–10, BALB.K mice. (C) 24 weeks after infection. Lanes 1–5, B10.BR mice; Lanes 6–10, BALB.K mice. (D) 32 weeks after infection. Lanes 1–4, B10.BR mice; Lanes 5–9, BALB.K mice. Control PCR reactions were performed with a different set of primers which amplify endogenous ecotropic (835 bp) virus as described under Material and Methods. An example of this reaction is shown in E (performed at 8 weeks after infection). Controls were performed for each time point. Positive controls (lane designated by +) were bone marrow cells from either a BALB.K or B10.BR mouse infected for 1 week with E-55+ MuLV since previous studies had determined that at this time point mice had high number of virus-infected cells. Negative controls (lane designated by −) were bone marrow cells from uninfected mice. MW, molecular weight markers, (Lambda DNA digested with *Eco*RI and *Hind*III). Data shown are representative of studies performed at least three times for each time point. In other experiments, extracts prepared from the bone marrow and spleens of E-55+ MuLV-infected mice that produced no 2 Kb E-55+ MuLV-specific band had virus titers of 0 focus forming units (FFU)/ml as measured in a fluorescent focus assay, whereas extracts from cells that produced this 2 kb band had virus titers between 2.8×10^3 and 2.7×10^5 FFU/ml.

mine the role of different lymphocyte populations in limiting the number of virus-infected cells in B10.BR bone marrow. In these experiments, bone marrow cells were

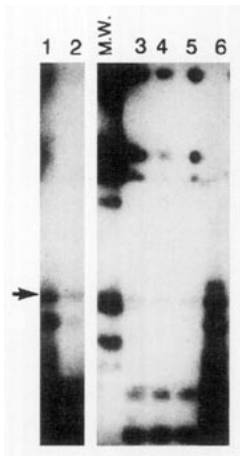

FIG. 2. Detection of E-55+ MuLV provirus by transfer of donor bone marrow cells from infected mice to lethally irradiated recipients. 5×10^6 bone marrow cells from B10.BR mice infected 8 weeks previously were treated with anti CD3 + C′ to remove T cells and inoculated iv into lethally irradiated (900R) syngeneic recipients. PCR amplification and Southern blot hybridization to detect provirus was performed as described in the legend to Fig. 1. Lanes 1 and 2, Bone marrow harvested from lethally irradiated mice 4 weeks after the transfer of anti-CD3 +C′-treated bone marrow from donors infected 8 weeks previously. Lane 3, Pooled bone marrow (untreated) from donor mice prior to transfer into recipients. Lane 4, Pooled spleen cells (untreated) from donor mice prior to transfer. Lane 5, uninfected bone marrow used as negative control. Lane 6, E-55+ MuLV-induced tumor cell line used as positive control. The expected 2 kb E-55+ MuLV-specific product is denoted with an arrow. Data shown are representative of four experiments.

harvested from E-55+ MuLV-infected mice, treated with anti-CD3 + C′ to remove T cells, and inoculated into lethally irradiated (900R) mice along with autologous donor spleen cells as a source of immune lymphocytes. Donor spleen cells were either untreated or treated prior to transfer with anti-CD3 + C′ to remove T cells or anti-Ia^k + C′ to remove B cells. At the time of inoculation into recipient mice neither infected donor spleen nor bone marrow cells demonstrated provirus detectable by PCR analysis (data not shown). Eight weeks after cell transfer, no provirus was detected in the bone marrow of recipients receiving infected bone marrow and untreated spleen cells (Fig. 4, lanes marked B cell +, CD4+, CD8+) or B-cell-depleted spleen cells (Fig. 4, lanes marked B

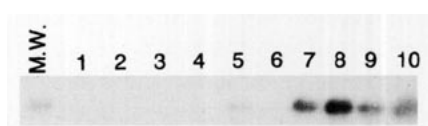

FIG. 3. Detection of provirus in normal and sublethally irradiated B10.BR mice 8 weeks after virus inoculation. Both normal and sublethally irradiated (550R) B10.BR mice were inoculated with 10^4 FFU per mouse. Eight weeks after inoculation, bone marrow cells were harvested and analyzed for the presence of provirus by PCR and Southern blot analysis as described in the legend to Figure 1. Lanes 1–5, Bone marrow from normal immunocompetent mice. Lanes 6–10, bone marrow from sublethally irradiated immunosuppressed mice. MW, molecular weight marker. Data shown are representative of two experiments.

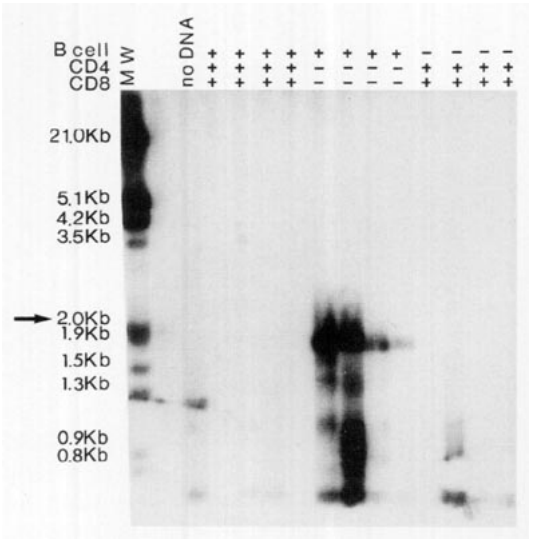

FIG. 4. Determination of the anti-virus effector cells in persistently infected B10.BR mice. Spleen cells from B10.BR donors that were infected 8 weeks previously with E-55+ MuLV were pooled and transferred into syngeneic recipient mice after antibody and C′ treatment and three cycles of panning as described under Material and Methods. 5×10^6 bone marrow cells from 12-week-infected mice were mixed with 5×10^6 spleen cells and injected iv into each recipient mouse. Recipient mice were lethally irradiated (900R) 24 h prior to spleen cell and bone marrow transfer. Three weeks after donor cell transfer, recipients were sacrificed and their bone marrow was harvested and analyzed for the presence of provirus by Southern analysis of PCR products as described in the legend to Fig. 1. The expected 2-kb E-55+ MuLV-specific product is marked with an arrow. (+++), mice that receive whole spleen cells from the infected donors; (---), mice that receive bone marrow from the infected donors; +, cells not depleted from the spleen cell population; −, cells depleted from spleen cell population. Data shown are representative of three experiments.

cell −, CD4 +, CD8 +). In contrast, provirus was readily detected in the bone marrow of mice that received T-cell-depleted spleen cells (Fig. 4, lanes marked B cell +, CD4 −, CD8 −). These results demonstrated that T cells and not B cells were responsible for maintaining virus-infected cells at low levels in these recipient mice.

Similar adoptive transfer studies in BALB.K mice demonstrated that these mice failed to generate the effective T cell response that effectively limited provirus expression in infected B10.BR mice (Fig. 5). Using the same protocols described above for the adoptive transfer studies in B10.BR mice, transfer of infected bone marrow cells along with untreated spleen cells (Fig. 5, lanes marked T +, B +) from the same donor failed to inhibit the expression of cells containing provirus. These results suggested that unlike B10.BR mice, BALB.K mice lack T effector cells that maintain virus-infected cells at low levels.

In all adoptive transfer experiments, the percentages of T cells was 26.0–36.6% of the B10.BR spleen cell population and 30.0–36.7% of the BALB.K population. Since the populations of cells from each strain transferred in each experiment were comparable, it appeared unlikely that numerical differences in the populations of cells transferred might account for the difference in containment of virus-infected cells observed between BALB.K and B10.BR.

Previous studies had determined that a non-MHC gene, *Rfv-3,* controlled resistance to Friend leukemia virus-induced leukemia by regulating antibody production against virus antigens (Doig and Chesebro, 1979; Chesebro *et al.,* 1979). Since resistance to E-55+ MuLV-induced lymphoma is also controlled by a non-MHC-linked gene(s) and the *env* regions of E-55+ MuLV and the F-MuLV component of FV are highly homologous (Tumas *et al.,* 1993b), it appeared that the differences between B10.BR and BALB.K related to resistance to E-55+ MuLV and containment of virus-infected cells might be the result, at least in part, of differences in the ability of these mice to produce anti-virus antibodies. Titers of anti-virus antibody were measured in the serum of BALB.K and B10.BR at various intervals after virus inoculation by complement-mediated cytotoxicity assays as described under Materials and Methods. No significant difference was found in the cytotoxic antibody titers between BALB.K and B10.BR 8 weeks after inoculation (Fig. 6). Serum from mice infected for 2 and 24 weeks were also tested and no difference in the anti-virus antibodies titers were found at either time point (data not shown). These results indicate that there is little difference in total anti-virus antibody produced by these strains at these time points.

DISCUSSION

In the studies described above, we have demonstrated that differences exist in the ability of susceptible BALB.K

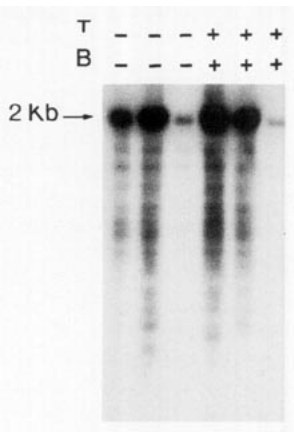

FIG. 5. Absence of the anti-virus effector cells in persistently infected BALB.K mice. Spleen cells from two infected donors inoculated 8 weeks previously with E-55+ MuLV were pooled and used in these transfer studies. 5×10^6 bone marrow cells alone (-- lanes) from infected donors or 5×10^6 bone marrow mixed with 5×10^6 spleen cells (++ lanes) from the same donors were injected iv into lethally irradiated (900R) mice. Three weeks after donor cell transfer, recipients were sacrificed and their bone marrow was harvested and analyzed for the presence of provirus by Southern blot analysis of PCR products as described in the legend to Fig. 1. Recipient groups include three mice per group. The expected 2-kb E-55+ MuLV-specific band is marked with an arrow. Data shown are representative of two experiments.

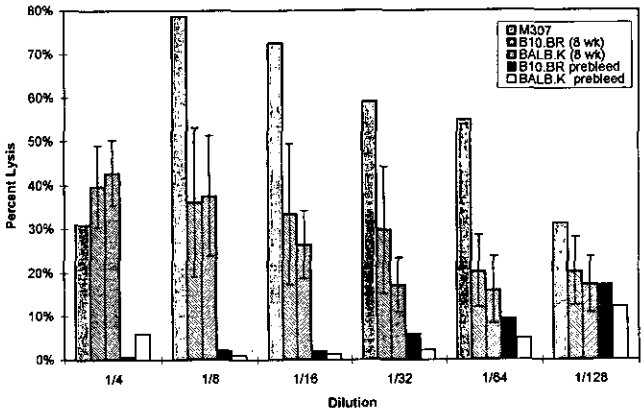

FIG. 6. Cytotoxic antibody titer in B10.BR and BALB.K mice 8 weeks after infection. Serial twofold dilution of sera from B10.BR and BALB.K mice infected for 8 weeks with E-55+ MuLV were tested for cytotoxic activity as described under Material and Methods. Average percentage lysis of each group is presented with standard error bars. Prebleed sera were pooled and used as negative control. Monoclonal antibody produced by the m307 hybridoma (Chesebro *et al.*, 1983, 1981), which binds E-55+ MuLV gp70, was used as a positive control. Data shown are representative of two experiments.

(progressors) and resistant B10.BR (long-term survivors) mice to generate an effective immune response that controls E-55+ MuLV infection and E-55+ MuLV-induced disease despite the fact that these animals express the same $H\text{-}2^k$ haplotype and both are persistently infected. Both BALB.K and B10.BR mice generate an effective early immune response against virus and/or virus-infected cells which leads to a dramatic decrease in the number of virus-infected cells 4–8 weeks after infection. However, in both strains this initial immune response fails to eliminate virus entirely resulting in a persistent infection. BALB.K mice subsequently progress to show an increase in the number of virus-infected cells associated with the development of lymphoma. In contrast, B10.BR mice fail to develop disease or an increase in virus-infected cells up to 24 months after infection.

Several mechanisms by which viruses evade an effective host immune response to establish persistent infections have been identified. For example, during the course of a virus infection, variant viruses that differ from the parental virus used for initial infection with respect to antigen expression may emerge. These types of antigenic variants have been identified in infection with various types of retroviruses, including equine infectious anemia virus (EIAV) (Montelaro *et al.*, 1984; Salinovich *et al.*, 1986; Payne *et al.*, 1987; Hussain and Issel, 1987) and HIV (Rowland-Jones *et al.*, 1992; Nara *et al.*, 1990). Equine infectious anemia is unique among retrovirus-induced diseases in that clinical symptoms and associated bursts of viremia occur in sequential episodes separated by several weeks or months. EIAV isolates taken from an infected pony after sequential disease episodes could be distinguished antigenically by neutralization assays with immune serum from the infected pony and by mAb against gp90, the major surface glycoprotein (Salinovich *et al.*, 1986; Hussain and Issel, 1987). These isolates also differed in biochemical alterations involving amino acid sequence and glycosylation patterns in the virion glycoproteins gp90 and gp45. Thus, it appeared that novel antigenic variants of EIAV predominated during each clinical episode and were responsible for persistent EIAV infection. Antigenic variants of HIV have also been isolated from infected individuals, which strongly suggests that the generation of these variants plays an important role in persistent HIV infection (Rowland-Jones *et al.*, 1992; Nara *et al.*, 1990; Coffin, 1986).

In a similar manner, previous studies from this laboratory have demonstrated that antigenic variant viruses arise in BALB.K mice infected with E-55+ MuLV associated with the development of lymphoma (Tumas *et al.*, 1993b). Sequence analysis of the *env* region of viruses isolated from lymphomatous spleens and a cultured lymphoma cell line derived from an E-55+ MuLV-infected mouse demonstrated that variant viruses are formed as the result of recombination between E-55+ MuLV and the *Emv-1* locus encoding endogenous ecotropic retrovirus (Jenkins *et al.*, 1982). However, despite the fact that a locus encoding a similar endogenous ecotropic retrovirus (*Emv-2*) is present in the genome of C57BL mice (Jenkins *et al.*, 1982) and *Emv-1* is present in (BALB.K × B10.BR)F1 mice, these mice are resistant to E-55+ MuLV-induced lymphomagenesis. Thus, it appears unlikely that the resistance to E-55+ MuLV-induced lymphoma in B10.BR mice can be explained by differences in the ability of the E-55+ MuLV to undergo antigenic variation by recombining with endogenous retrovirus sequences.

Viruses may also persistently infect a host by causing the abrogation of effective anti-virus immune responses. The long-term survival of E-55+ MuLV-infected B10.BR mice appears to be the result of a late T cell response which is not generated in BALB.K mice. Since both strains of mice generate effective early anti-virus immune responses that lead to the decrease in numbers of virus-infected cells after acute infection, this late protective T cell response in B10.BR mice appears to be important in containing the persistent E-55+ MuLV infection at low levels. Thus, in the absence of this late T cell response, as observed in BALB.K mice, virus and/or virus-infected cells emerge to cause disease. One possible explanation for the lack of a late protective T cell response in BALB.K mice is that T cell exhaustion occurs in a manner similar to that observed in Lymphocytic choriomeningitis virus (LCMV) infection (Moskophidis *et al.*, 1993; Moskophidis *et al.*, 1994). LCMV infection results in the development of an anti-virus cytotoxic T cell response that causes the death of infected mice as the result of the immune-mediated destruction of virus-infected cells in the central nervous system. However, infection of mice with a variant

of LCMV, DOCILE, can result in the exhaustion of anti-LCMV cytotoxic T cells. In addition, both *H-2*-linked and non-*H-2*-linked genes may affect the exhaustion of cytotoxic cells as well (Moskophidis *et al.*, 1994). This abrogation of the cytotoxic T cell response results in a persistent infection in which virus was isolated from the spleen and thymus of infected mice for 200 days after infection although no disease was observed (Moskophidis *et al.*, 1993).

Alternatively, the late effective T cell response observed in B10.BR mice may not occur in BALB.K mice as the consequence of genetic differences between the two strains of mice that results in differences in the generation of Th1- and Th2-type responses. Th1-type responses result in strong cell-mediated immune responses, whereas predominant Th2-type responses are characterized by reduced or undetectable cell-mediated responses (Fitch *et al.*, 1993). Thus, generation of Th1-type response in virus-infected B10.BR mice might promote an effective late T cell response, whereas this response might not occur in BALB.K if these mice generate a Th2-type response upon virus infection. Differences between strains of mice with respect to their ability to generate Th1-type and Th2-type responses have been previously been observed in mice infected with another murine retrovirus, LP-BM5 (Gazzinelli *et al.*, 1992).

The E-55+ MuLV used in these studies has been found to be highly homologous to F-MuLV, the replication competent helper virus component of the FV complex, with respect to sequence analysis of the *env* regions of these viruses (Tumas *et al.*, 1993b). Previous studies have identified genes responsible for differences in susceptibility to FV-induced leukemia (Chesebro *et al.*, 1990). Two genes, *Rfv-1* and *Rfv-2,* have been found to be linked to the *H-2* complex and appear to regulate the cell-mediated immune response to virus antigen (Chesebro and Wehrly, 1978; Britt and Chesebro, 1980; Miyazawa *et al.*, 1992a, b). A third gene, *Rfv-3,* is not linked to *H-2* and plays a role in the regulation of the antibody response to FV (Doig and Chesebro, 1979; Chesebro *et al.*, 1979). The results of our studies strongly suggest that none of these previously identified genes plays a role in the difference between the E-55+ MuLV resistant and susceptible strains used. Both BALB.K and B10.BR mice express the $H\text{-}2^k$ haplotype and are, therefore, identical with respect to *Rfv-1* and *Rfv-2.* In addition, although strains differ with respect to their alleles expressed at *Rfv-3,* we have not been able to find differences between their antibody response to E-55+ MuLV. It, therefore, appears that a previously unidentified non-*H-2*-linked gene(s) in the genetic background of these strains is responsible for the difference in their susceptibility to E-55+ MuLV.

Common patterns between the course of E-55+ MuLV infection in mice described in this report and the previously described course of HIV infection in humans are notable. Primary HIV and E-55+ MuLV infections are associated with high levels of virus expression followed shortly by the generation of anti-virus immune responses that result in a dramatic decline in virus infection (Pantaleo *et al.*, 1993a; Daar *et al.*, 1991; Clark *et al.*, 1991; Tumas *et al.*, 1993a). Especially noteworthy is the similarity in both virus infections with respect to the persistence of retrovirus infection at low levels despite an ongoing anti-virus immune response. Recent studies have indicated that long-term survival in HIV-infected individuals is a function of the expression of certain genes (Ezzell, 1993). Rapid progression and long-term survival in HIV-infected individuals appear to be associated with the expression of certain *HLA* alleles (Steel *et al.*, 1988; Kaslow and Mann, 1994). These studies strongly suggest that a host genetic component(s) is involved in long-term survival, and the association with the major histocompatibility complex suggests the possibility of immune response differences between the two cohorts. The similarities between phenomena associated with HIV and E-55+ MuLV infections raises the possibility that differences between long-term survivors and rapid progressors in HIV infection may be analogous to differences between E-55+ MuLV susceptible (BALB.K) and resistant (B10.BR) mice. Therefore, analysis of the genetic basis for immune-mediated resistance to E-55+ MuLV infection may identify non-*H-2*-linked genes and/or immunological events that are important for long-term survival in HIV infection.

REFERENCES

Britt, W. J., and Chesebro, B. (1980). *H-2D (Rfv-1)* gene influence on recovery from Friend virus leukemia is mediated by nonleukemic cells of the spleen and bone marrow. *J. Exp. Med.* **152,** 1795–1804.

Chesebro, B., Wehrly, K., Doig, D., and Nishio, J. (1979). Antibody-induced modulation of Friend virus cell surface antigens decreases virus production by persistent erythroleukemia cells: influence of the *Rfv-3* gene. *Proc. Natl. Acad. Sci. USA* **76,** 5784–5788.

Chesebro, B., Wehrly, K., Cloyd, M., Britt, W., Portis, J., Collins, J., and Nishio, J. (1981). Characterization of mouse monoclonal antibodies specific for Friend murine leukemia virus-induced erythroleukemia cells: Friend-specific and FMR-specific antigens. *Virology* **112,** 131–144.

Chesebro, B., Britt, W., Evans, L., Wehrly, K., Nishio, J., and Cloyd, M. (1983). Characterization of monoclonal antibodies reactive with murine leukemia viruses: Use in analysis of strains of Friend MCF and Friend ecotropic murine leukemia virus. *Virology* **127,** 134–148.

Chesebro, B., Miyazawa, M., and Britt, W. J. (1990). Host genetic control of spontaneous and induced immunity to Friend murine retrovirus infection. *Annu. Rev. Immunol.* **8,** 477–499.

Chesebro, B., and Wehrly, K. (1978). *Rfv-1* and *Rfv-2,* two *H-2*-associated genes that influence recovery from Friend leukemia virus-induced splenomegaly. *J. Immunol.* **120,** 1081–1085.

Chesebro, B., and Wehrly, K. (1979). Identification of a non-*H-2* gene *(Rfv-3)* influencing recovery from viremia and leukemia induced by Friend virus complex. *Proc. Nat. Acad. Sci. USA* **76,** 425–429.

Clark, S. J., Saag, M. S., Decker, W. D., Campbell-Hill, S., Roberson, J. L., Veldkamp, P. J., Kappes, J. C., Hahn, B. H., and Shaw, G. M. (1991). High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection. *New Engl. J. Med.* **324,** 954–960.

Coffin, J. M. (1986). Genetic variation in AIDS viruses. *Cell* **46,** 1–4.

Daar, E. S., Moudgil, T., Meyer, R. D., and Ho, D. D. (1991). Transient high levels of viremia in patients with primary human immunodeficiency virus type 1 infection. *New Eng. J. Med.* **324,** 961–964. [See comments]

Doig, D., and Chesebro, B. (1979). Anti-Friend virus antibody is associated with recovery from viremia and loss of viral leukemia cell-surface antigens in leukemic mice. Identification of Rfv-3 as a gene locus influencing antibody production. *J. Exp. Med.* **150,** 10–19.

Ezzell, C. (1993). On Borrowed time:Long-term survivors of HIV-1 infection. *J. NIH Res.* **5**(7), 77–82.

Fitch, F. W., McKisic, M. D., Lancki, D. W., and Gajewski, T. F. (1993). Differential regulation of murine T lymphocyte subsets. *Annu. Rev. Immunol.* **11,** 29–48.

Gazzinelli, R. T., Makino, M., Chattopadhyay, S. K., Snapper, C. M., Sher, A., Hugin, A. W., and Morse, H. C. (1992). CD4+ subset regulation in viral infection. Preferential activation of Th2 cells during progression of retrovirus-induced immunodeficiency in mice. *J. Immunol.* **148,** 182–188.

Hussain, K. A., and C. J. Issel (1987). Antigenic analysis of equine infectious anemia virus (EIAV) variants by using monoclonal antibodies: Epitopes of glycoprotein gp90 of EIAV stimulate neutralizing antibodies. *J. Virol.* **61,** 2956–2961.

Jenkins, N. A., Copeland, N. G., Taylor, B. A., and Lee, B. K. (1982). Organization, distribution, and stability of endogenous ecotropic murine leukemia virus DNA sequences in chromosomes of Mus musculus. *J. Virol.* **43,** 26–36.

Kaslow, R. A., and Mann, D. L. (1994). The role of the major histocompatibility complex in human immunodeficiency virus infection--ever more complex? *J. Infect. Dis.* **169,** 1332–1333.

Klein, J. (1973). List of congenic lines of mice. I. Lines with differences at alloantigen loci. *Transplantation* **15,** 137–153.

Klyczek, K. K., and Blank, K. J. (1989). Novel class I-like molecule expressed on a murine leukemia virus-transformed cell line. *Cell. Immunol.* **118,** 222–228.

Miyazawa, M., Nishio, J., Wehrly, K., David, C. S., and Chesebro, B. (1992a). Spontaneous recovery from Friend retrovirus-induced leukemia. Mapping of the Rfv-2 gene in the Q/TL region of mouse MHC. *J. Immunol.* **148,** 1964–1967.

Miyazawa, M., Nishio, J., Wehrly, K., Jay, G., Melvold, R. W., and Chesebro, B. (1992b). Detailed mapping of the Rfv-1 gene that influences spontaneous recovery from Friend retrovirus-induced leukaemia. *Eur. J. Immunogenet.* **19,** 159–164.

Montelaro, R. C., Parekh, B., Orrego, A., and Issel, C. J. (1984). Antigenic variation during persistent infection by equine infectious anemia virus, a retrovirus. *J. Biol. Chem.* **259,** 10539–10544.

Moskophidis, D., Lechner, F., Pircher, H., and Zinkernagel, R. M. (1993). Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature* **362,** 758–761.

Moskophidis, D., Lechner, F., Hengartner, H., and Zinkernagel, R. M. (1994). MHC class I and non-MHC-linked capacity for generating an anti-viral CTL response determines susceptibility to CTL exhaustion and establishment of virus persistence in mice. *J. Immunol.* **152,** 4976–4983.

Nara, P., Smit, L., Dunlop, N., Hatch, W., Merges, M., Waters, D., Kelliher, J., Krone, W., and Goudsmit, J. (1990). Evidence for rapid selection and deletion of HIV-1 subpopulations in vivo by V3-specific neutralizing antibody: A model of humoral-associated selection. *Dev. Biol. Stand.* **72,** 315–341.

Pan, L. Z., Werner, A., and Levy, J. A. (1993). Detection of plasma viremia in human immunodeficiency virus-infected individuals at all clinical stages. *J. Clin. Microbiol.* **31,** 283–288.

Pantaleo, G., Graziosi, C., and Fauci, A. S. (1993a). New concepts in the immunopathogenesis of human immunodeficiency virus infection. *New Engl. J. Med.* **328,** 327–335.

Pantaleo, G., Graziosi, C., Demarest, J. F., Butini, L., Montroni, M., Fox, C. H., Orenstein, J. M., Kotler, D. P., and Fauci, A. S. (1993b). HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature* **362,** 355–358.

Payne, S. L., Fang, F. D., Liu, C. P., Dhruva, B. R., Rwambo, P., Issel, C. J., and Montelaro, R. C. (1987). Antigenic variation and lentivirus persistence: Variations in envelope gene sequences during EIAV infection resemble changes reported for sequential isolates of HIV. *Virology* **161,** 321–331.

Piatak, M., Jr., Saag, M. S., Yang, L. C., Clark, S. J., Kappes, J. C., Luk, K. C., Hahn, B. H., Shaw, G. M., and Lifson, J. D. (1993). High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science* **259,** 1749–1754.

Portoles, P., Rojo, J., Golby, A., Bonneville, M., Gromkowski, S., Greenbaum, L., Janeway, C. A., Jr., Murphy, D. B., and Bottomly, K. (1989). Monoclonal antibodies to murine CD3 epsilon define distinct epitopes, one of which may interact with CD4 during T cell activation. *J. Immunol.* **142,** 4169–4175.

Pozsgay, J. M., Klyczek, K. K., and Blank, K. J. (1989). In vivo generation of antigenic variants of murine retroviruses. *Virology* **173,** 330–334.

Rowland-Jones, S. L., Phillips, R. E., Nixon, D. F., Gotch, F. M., Edwards, J. P., Ogunlesi, A. O., Elvin, J. G., Rothbard, J. A., Bangham, C. R., Rizza, C. R., *et al.* (1992). Human immunodeficiency virus variants that escape cytotoxic T-cell recognition. *AIDS Res. Hum. Retroviruses* **8,** 1353–1354.

Salinovich, O., Payne, S. L., Montelaro, R. C., Hussain, K. A., Issel, C. J., and Schnorr, K. L. (1986). Rapid emergence of novel antigenic and genetic variants of equine infectious anemia virus during persistent infection. *J. Virol.* **57,** 71–80.

Steel, C. M., Ludlam, C. A., Beatson, D., Peutherer, J. F., Cuthbert, R. J., Simmonds, P., Morrison, H., and Jones, M. (1988). HLA haplotype A1 B8 DR3 as a risk factor for HIV-related disease. *Lancet* **1,** 1185–1188.

Tumas, K., Overmoyer, B., Clevenger, C. V., Blank, K. J., and Prystowsky, M. B. (1993a). Murine leukemia virus infection in immunocompetent adult mice. *Virology* **192,** 1–10.

Tumas, K. M., Poszgay, J. M., Avidan, N., Ksiazek, S. J., Overmoyer, B., Blank, K. J., and Prystowsky, M. B. (1993b). Loss of antigenic epitopes as the result of *env* gene recombination in retrovirus-induced leukemia in immunocompetent mice. *Virology* **192,** 587–595.